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Oral immunization of mice with transgenic tomato fruit expressing respiratory syncytial virus-F protein induces a systemic immune response

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Abstract

Respiratory syncytial virus (RSV) is one of the most important pathogens of infancy and early childhood. Here a fruit-based edible subunit vaccine against RSV was developed by expressing the RSV fusion (F) protein gene in transgenic tomato plants. The F-gene was expressed in ripening tomato fruit under the control of the fruit-specific E8 promoter. Oral immunization of mice with ripe transgenic tomato fruits led to the induction of both serum and mucosal RSV-F specific antibodies. The ratio of immunoglobulin subclasses produced in response to immunization suggested that a type 1 T-helper cell immune response was preferentially induced. Serum antibodies showed an increased titer when the immunized mice were exposed to inactivated RSV antigen.

Introduction

Respiratory syncytial virus (RSV) is an enveloped virus of the genus *Pneumovirus* (Hall, 1992). It infects virtually all children worldwide and is one of the most important pathogens of infancy and early childhood and a major cause of serious lower respiratory tract diseases (Hall, 1992). Surveys of children hospitalized with RSV show mortality rates of 0.1–2.5% (McIntosh & Chanock, 1990; Prober & Wang, 1997). Re-infection with RSV is also common because natural infection confers only temporary protection against the disease. The immunity elicited by primary infection declines rapidly and cannot prevent reinfection although the severity of the disease may decrease (Hall, 1992). In recent years, RSV infections have been increasingly noted in nursing homes and other group settings serving the institutionalized elderly (Hall, 1992; Fleming & Cross, 1993; Falsey & Walsh, 1998) with pneumonia developing in up to two-thirds of the patients (DeVincenzo, 1997).

Though multiple attempts have been made to produce an effective vaccine that affords long-lasting protection against RSV, so far none have been successful (DeVincenzo, 1997; Coffin & Offit, 1997). Early attempts to use formalin-inactivated RSV (FI-RSV) as a vaccine resulted in more severe lower respiratory tract disease and higher mortality rates in response to RSV infection in immunized children than non-immunized children (Kim et al., 1969). Analysis of the immune responses of the two groups to RSV infection showed that a single immunization with FI-RSV resulted in the recruitment of large numbers of eosinophils to infected lung tissues, which led to inflammatory responses. In contrast, prior infection with RSV often produced a

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cellular immune response that cleared RSV from the lung with little inflammation (Hall, 1994). The response induced by FI-RSV is characteristic of a type 2 T-helper cell (Th2) response (Srikiatkhachorn and Braciale, 1997a).

Oral exposure to particulate and replicating antigen can induce protective immune responses. However, exposure to soluble protein antigens in food most commonly results in immunological tolerance rather than protective immunity (Strobel & Mowat, 1998). Even so, very low doses of soluble protein antigens have been shown to produce systemic responses (Strobel & Mowat, 1998). This phenomenon may be at least partially responsible for the successful elicitation of serum and mucosal immune responses to recombinant antigens in transgenic plants (Haq et al., 1995; Mason et al., 1996; Arakawa et al., 1998). These observations have led to the suggestion that oral immunization with antigens expressed in transgenic plants can be useful against some disease-causing agents (Arntzen, 1997). Edible plant-derived vaccines have elicited the production both serum IgG- and mucosal IgA-specific antibodies in mice (Haq et al., 1995; Mason et al., 1996; Arakawa et al., 1998) and humans (Tacket et al., 1998). The use of plants for expression and delivery of recombinant proteins is an attractive alternative for developing vaccines. A main advantage of an edible plant-derived vaccine is that it is delivered as a part of the diet. Further, purification to remove viral contaminants is not necessary since plant viruses do not infect humans.

The glycoproteins exposed on the surface of the RSV envelope are potential candidates as immunogens for development of an RSV vaccine. F and G are the two largest glycoproteins in the envelope. The 90-kDa G glycoprotein is responsible for the initial attachment of RSV to target cells (Levine et al., 1987). The 68-kDa fusion protein (F) enables the lipid membrane of the virus to fuse with the lipid membrane of target cells, thus allowing the viral RNA to be inserted into the target cells (Walsh et al., 1987). Variant RSV strains are known and fall into two main groups, A and B, which differ mainly in the G protein. The F protein, however, is well conserved with 89% amino-acid identity between groups A and B (Johnson & Collins, 1988). Antibodies to the F protein are cross-reactive between the two groups (Anderson et al., 1985). Immunization of BALB/c mice with recombinant vaccinia viruses expressing the RSV-F protein resulted in the production of neutralizing antibodies (Alwan & Openshaw, 1993) that protected against RSV infection (Gaddum et al., 1996). In contrast, immunization with recombinant vaccinia viruses expressing the RSV-G resulted in severe eosinophilia and hypersensitivity in mice infected with RSV (Srikiatkhachorn & Braciale, 1997b). Therefore, the F protein was chosen here for the development of an edible oral vaccine for RSV. We tested the ability of genetically engineered tomato fruit to serve as a novel delivery system for oral immunization of BALB/c mice. The immunized mice produced both serum and mucosal antibodies against the RSV-F antigen.

Materials and methods

Chimeric gene constructs

Plasmids containing the RSV-F coding sequence downstream of the constitutively expressed cauliflower mosaic virus (CaMV) 35S promoter and the preferentially fruit-expressed E8 promoter were constructed as follows. The A2 F11 cDNA copy of the RSV-F coding sequence (Olmsted et al., 1986) (gift from Dr. P. Collins, National Institutes of Health, Bethesda, MD) was amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Roche Molecular Biochemicals, USA). The forward and reverse primers were designed to complement the RSV-F start initiation and termination codons, respectively (Sandhu et al., 1999). The amplified cDNA was ligated into pBlueScript II KS- (Stratagene, USA) opened at *Not*I and *Sst*I sites, and its sequence was confirmed. The RSV-F fragment was cleaved from pBlueScript II KS- with *Xba*I and *Sst*I and ligated into the binary plant vector pBI121 (Clontech, USA) where it replaced the constituent *gusA* gene and yielded pJSS3 (Figure 1). The 2.2-kbp E8 promoter from tomato (Deikman et al., 1992) (gift from Dr. R. Fischer, University of California, Berkeley) was cleaved from the pUC118 plasmid with *Eco*RI and *Bam*HI and ligated into pBlueScript II KS- opened at these restriction sites. This provided a *Hin*dIII site at the 5′-end of the E8 promoter. The CaMV 35S promoter upstream of the RSV-F gene in pJSS3 (Figure 1) was removed by *Hin*dIII and *Bam*HI digestion and replaced by the E8 promoter to give pJSS4 (Figure 1).

Tomato transformation

Plasmids pJSS3 and pJSS4 were introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Koncz & Schell, 1986) (gift from Dr. S. Gelvin,

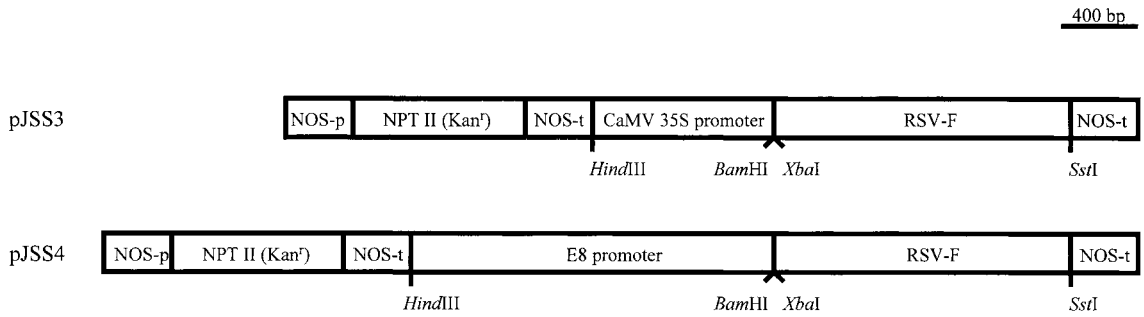

Figure 1. Plasmids containing the RSV-F coding-sequence (cDNA) under the transcriptional control of either the CaMV 35S or E8 promoter. Restriction enzyme sites used for making the constructs are indicated. Plasmids are designated on the left and are shown in the 5′ to 3′ orientation. NOS-p: nopaline synthase promoter; NOS-t: nopaline synthase terminator; NPT II: neomycin phosphotransferase II.

Purdue University, IN) via electroporation and used to transform cherry tomato (*Lycopersicon esculentum* Mill. Cv. Swifty Belle) following modification of protocols described by Bird et al. (1988) and Hamza and Chupeau (1993). Eight-days post germination cotyledons were excised from *in vitro*-germinated seedlings, and cocultivated for 48 h with an overnight-grown culture of *A. tumefaciens* carrying the plasmids on a cocultivation medium consisting of Murashige and Skoog (MS) salts, Gamborg's B5 vitamins, and supplemented with 1 mg/l α-naphthaleneacetic acid, 1 mg/l thidiazuron (TDZ), 640 mg/l 2-[*N*-morpholino]ethane sulfonic acid, 30 g/l sucrose, and 6.5 g/l Difco bacto-agar. The pH of the medium was adjusted to 6.1 with 1N NaOH. Cotyledons were then rinsed with sterilized de-ionized water, blotted dry on a sterilized paper towel, and placed onto a selection medium consisting of MS salts and Gamborg's B5 vitamins, and supplemented with 0.5 mg/l 6-benzyladenine, 1 mg/l TDZ, 0.5 mg/l 3-indoleacetic-acid, 100 mg/l kanamycin, 500 mg/l carbenicillin, and 6.5 g/l Difco bacto-agar. The pH was adjusted to 5.8 with 1N NaOH. After six weeks, kanamycin-resistant shoot regenerants were removed from callus, and transferred to a rooting medium consisting of MS salts with Gamborg's B5 vitamins, 30 g/l sucrose, and 6.5 g/l Difco bacto-agar (pH adjusted to 5.8). Rooted plantlets were acclimatized and transferred to a greenhouse for fruiting. Transgenic plants were checked for the presence of transgene(s) in leaf tissue using Southern blot analysis (Sambrook et al., 1989).

Analysis of transformed tomato fruits for RSV-F protein

To estimate the amount of RSV-F expressed in transgenic fruit, an RSV-F-protein standard curve was derived as follows. The level of F was determined in an available 'RSV antigen' (Cat. No. R02712, Biodesign International, USA) since purified F-protein was not available commercially. Two microlitres (20 µg) of the antigen were mixed with 2 µl of SDS-PAGE loading buffer (Sambrook et al., 1989). The mixture was heated at 95°C for 5 min and separated on an SDS-polyacrylamide gel consisting of a stacking gel of 5% (w/v) and a resolving gel of 12% (w/v) acrylamide (Sambrook et al., 1989). The gel was electrophoresed at 100V for 2 h, stained with Coomassie brilliant blue for 15 min and destained for 10 h. The level of F (68-kDa band on the gel) in the RSV antigen was 48% as determined densitometeric scanning of the resolved proteins. Ripe wild-type tomato fruit was homogenized with a mortar and pestle in 3 volumes (w/v) of coating buffer (Clark & Adams, 1977). Amounts of 'RSV antigen' containing 0–40 µg of RSV-F were diluted in the homogenate and 100 µl was used to coat each well of an enzyme-linked immunosorbent assay (ELISA) microtiter plate (Immulon 1B, Dynex Technologies, USA) and analyzed as described by Clark and Adams (1977).

Transformed tomato fruits also were homogenized in 3 volumes (w/v) of coating buffer and 100 µl was used to coat each well of an ELISA microtiter plate. Monoclonal IgG antibody against the RSV-F protein (Cat. No. MCA490, Serotec, USA) was diluted 1:1000, and 50 µl was added to each well. Secondary labeling was done using an alkaline phosphatase-conjugated rabbit anti-mouse IgG (Cat. No. A-1902, Sigma, USA) at a 1:1000 dilution with 50 µl added to each well. The label was detected by adding 50 µl of *p*-nitrophenyl phosphate to each well, and ELISA readings were recorded at 405 nm in a microplate autoreader (MRX, Dynex Technologies). These ELISA readings were compared with those obtained

with RSV-F to estimate the amount of recombinant RSV-F expressed in transgenic tomato fruit.

Protein extraction, gel electrophoresis, electroblotting, and immunoblot assay

Proteins were extracted from leaf, stem, root and fruit tissue of tomato plant 120 which showed the highest RSV-F expression, precipitated using acetone and resuspended in extraction buffer as described by Mason et al. (1992). An equal volume of SDS-PAGE loading buffer was added to each 25 µl (25 µg) protein sample. The mixture was heated at 95°C for 5 min and separated on SDS-polyacrylamide gels consisting of stacking gels of 5% (w/v) and resolving gels of 15% (w/v) acrylamide (Sambrook et al., 1989). Gels were electrophoresed at 100 V for 2–3 h until the dye front was about 5 mm from the bottom of the gel. Proteins were electrophoretically transferred to 0.45 µm nitrocellulose membranes for 15 min at 30 V using a semi-dry transfer apparatus (BioRad, USA) according to the manufacturer's instructions. Transferred proteins were subjected to primary labeling with a 1:1000 dilution of monoclonal antibodies against RSV-F (Cat. No. MCA490, Serotec), followed by secondary labeling with a 1:3000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate (Cat. No. 170-6412, BioRad). The label was analyzed using an Immunoblot assay kit (BioRad) according to the manufacturer's instructions.

Oral immunization with RSV-F

Twenty-five four-week-old female BALB/c mice were fed ripe tomato fruit tissue from plant line 120. Five control mice were fed ripe wild-type cherry tomato. The animals consumed approximately 60% of the fruit mass including pulp, skin, juice and seeds within 4 h. A total of five feedings were done on days 0, 4, 14, 18 and 28 essentially as described by Haq et al. (1995). Before the first feeding, transgenic fruits were harvested and stored at 4°C while protein determinations were conducted. For subsequent feedings, fruits were harvested and fed within 2 h.

Antibody assays

Animals were bled on day 32 as described by Haq et al. (1995) and sera were stored at −20°C until assayed. On day 33, the mice were given a single intramuscular injection (50 µl) of RSV antigen (Biodesign International) in phosphate-buffered saline at 12 mg/ml. All the mice were euthanized on day 39 and their sera and intestinal extracts assayed for antibodies against RSV-F. The small intestine from the duodenum to the ileal-cecal junction was excised to determine the presence of secreted mucosal IgA antibodies. The excised tissue was washed thoroughly with buffer (0.05 M Tris, 0.001 M EDTA, 0.003 M NaN_3, 0.2 M NaCl, pH 7.5) before preparing mucosal extracts according to Clements et al. (1986).

Antibody titers were analyzed by ELISA for production of antibodies to RSV-F. The serum and mucosal samples drawn from the preimmunized and immunized mice as well as those boosted with RSV antigen were analyzed by endpoint titer dilutions, and 50 µl was added to each well of an ELISA microtiter plate coated with 12 µg/ml of RSV antigen in coating buffer (Clark & Adams, 1977). Secondary labeling was done using alkaline phosphatase-conjugated antibodies specific for mouse IgG (Cat. No. A-1902, Sigma), IgA (Cat. No. A-4937, Sigma), IgG_1 (Cat. No. 61-0122, Zymed, South San Francisco, CA), IgG_{2a} (Cat. No. 61-0222, Zymed) or IgG_{2b} (Cat. No. 61-0322, Zymed) at a 1:1000 dilution. Standard curves using serial dilutions of purified mouse IgG_1 (Cat. No. 02-6100, Zymed), IgG_{2a} (Cat. No. M-5409, Sigma) and IgG_{2b} (Cat. No. M-5534, Sigma) showed that avidities of the antibodies for their specific IgG-subclass were indistinguishable. Labeling was detected with *p*-nitrophenol production and the absorbance was quantified at 405 nm (Clark & Adams, 1977). Individual samples were replicated three times to confirm the results. Wells were washed six times with deionized water and absorbances were calculated after subtraction of background values obtained in wells coated with buffer. Antibody titers were calculated by comparing absorbance values of serially diluted serum of control and immunized mice. The endpoint titers were those dilutions at which the mean absorbance readings obtained from control and immunized mice were not significantly different.

Statistics

Significant differences between antibody endpoint titers were determined by the non-paired *t*-test.

Results

Generation of transgenic tomato plants producing RSV-F antigen

The gene from human RSV encoding the F protein was placed under the transcriptional control of either the

constitutively expressed CaMV 35S promoter (Benfey et al., 1989) or the fruit specific E8 promoter (Lincoln et al., 1987) in gene constructs pJSS3 and pJSS4 (Figure 1). Through *Agrobacterium*-mediated transformation, 30 independent transgenic tomato plants were generated with each construct and confirmed by Southern blotting (data not shown). ELISA analysis demonstrated that recombinant RSV-F antigen was expressed in the ripe tomato fruit. Sixteen transgenic plants containing pJSS3 and 19 containing pJSS4 expressed the RSV-F antigen in the fruit at levels ranging from 1.0 to 32.5 μg/g fruit fresh weight. However, the average levels of RSV-F antigen in fruits of transgenic plants transformed with constructs driven by the E8 and CaMV 35S promoters were similar, 12.68 ± 2.55 and 9.01 ± 1.87 μg/g fruit fresh weight, respectively. For each gene construct, the five transgenic plants expressing the highest levels of the F-protein in their fruits were selected. The F-protein level for one representative fruit from each of the five tomato lines is presented in Figure 2. The transgenic plant designated as 120 accumulated the highest amount of RSV-F protein in its fruit (32.5 μg/g fruit fresh weight). The levels of F-protein varied among different fruits of the same plant. For example, 10 random fruits from plant 124 and plant 120 showed levels with standard deviations of 12% and 3% of the respective mean values.

Immunoblot analysis of proteins extracted from fruit, leaf, stem and root tissues of tomato plant

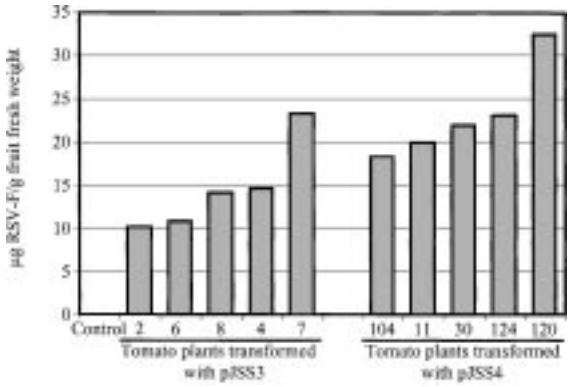

Figure 2. Amount of recombinant RSV-F antigen in ripe fruits of transgenic tomato plants transformed with the pJSS3 (CaMV 35S promoter) and pJSS4 (E8 promoter) constructs (Figure 1). For each construct five transgenic plants expressing the highest levels of RSV-F antigen in fruits were chosen. Results are shown for one representative fruit from each of the five tomato plants. Ripe fruit from wild-type tomato was used as a control. The numbers on the bottom of the figure represent the different tomato plants.

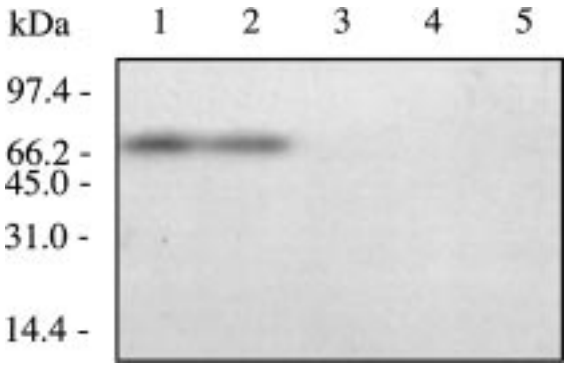

Figure 3. Immunoblot analysis of proteins extracted from transgenic tomato line 120 transformed with the pJSS4 construct containing the E8 promoter (Figure 1). Lane 1: RSV antigen; Lane 2: Proteins from fruit tissue; Lanes 3–5: Proteins from leaf, stem and root tissues, respectively. Lanes 2–5: 25 μg total protein from each tissue was loaded onto the gel.

120 using a monoclonal antibody specific for RSV-F showed specific binding only in the fruit tissue with a 68-kDa protein that comigrated with RSV antigen (Figure 3). This demonstrated the fruit-specific expression of RSV-F cDNA under the control of the E8 promoter. Thus, expression of a protein of the expected molecular mass and antigenicity in the tomato fruits suggested their possible use as edible vaccines.

Induction of both serum and mucosal antibodies and an immune response against RSV

Tomato fruit from line 120 was orally fed to 25 mice at five times during a 28-day period to test the ability of the expressed RSV-F protein to induce mucosal and serum responses. For each feeding, each mouse was given 5–7 g of ripe tomato fruit containing recombinant RSV-F protein and consumed 3–4 g. RSV-F-specific antibody induction was determined using ELISA on serum collected on day 32 from preimmunized, immunized and control mice (fed ripe wild-type tomato). The control and the preimmunized mice did not produce detectable anti-RSV-F antibodies (Figure 4A). Among the 25 orally-immunized mice, 22 showed significant ($P \leq 0.05$) serum IgG and IgA responses and produced anti-RSV-F antibodies. Figure 4A shows the mean serum IgG and IgA anti-RSV-F antibody titers in all 25 mice. There was no significant difference between the serum IgG and IgA endpoint titers. This result demonstrated successful oral immunization of mice and showed that fruit-derived RSV-F was active as an oral immunogen.

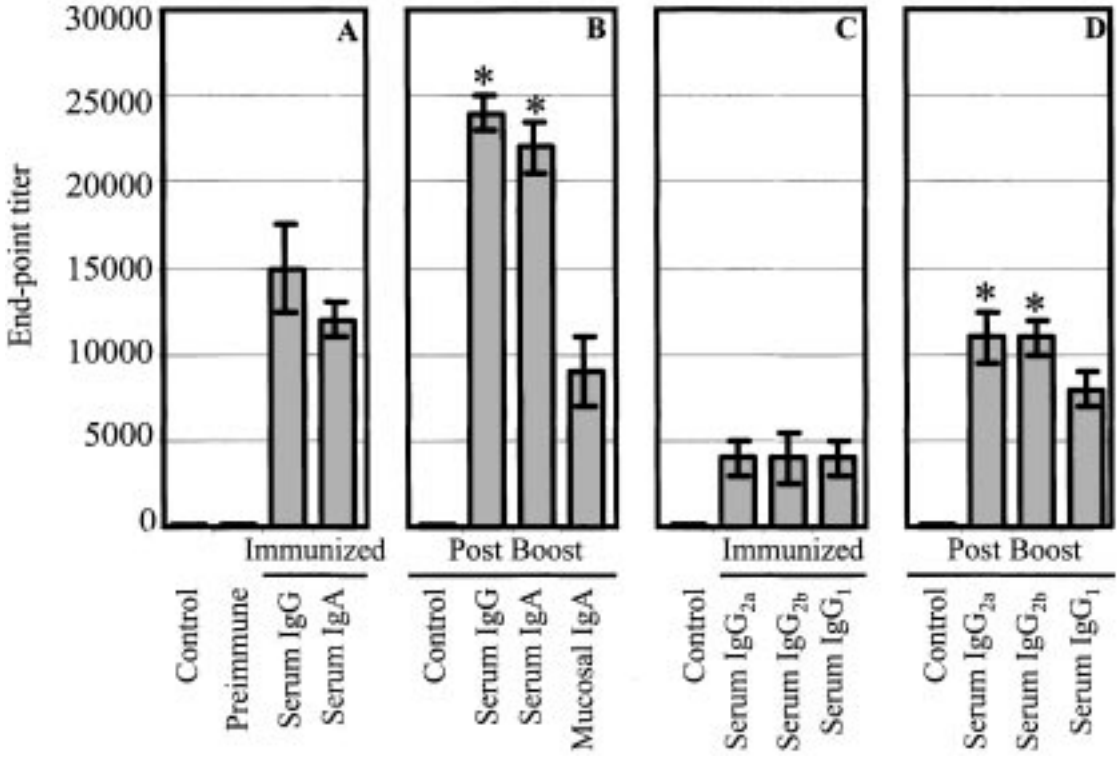

Figure 4. Serum and mucosal RSV-F specific antibody titers from mice fed transgenic or control tomatoes. Triplicate samples were serially diluted and measured by ELISA. Endpoint titers are plotted (mean ± SE) on a linear scale of the dilution factor. (A) RSV-F-specific serum IgG and IgA titers from samples collected prior to oral immunization (preimmune) and, on day 32, from control (fed wild-type tomato) and immunized mice (fed transgenic tomatoes). (B) RSV-F-specific serum IgG and IgA and mucosal (small intestine) IgA titers from samples collected from the same mice on day 39 after intramuscular injection (boost) on day 33 with inactivated RSV antigen. $^*P \leqslant 0.05$ vs. the immunized serum IgG and IgA samples. (C) RSV-F-specific serum titers of the three IgG sub-classes (IgG_{2a}, IgG_{2b} and IgG_1) from samples collected on day 32 from control and orally-immunized mice. (D) RSV-F-specific serum titers of the three IgG sub-classes from samples collected on day 39 from control mice and those boosted with inactivated RSV. $^*P \leqslant 0.05$ vs. the post-boost serum IgG_1.

To test their response to an inactivated RSV antigen, mice were boosted on day 33 with RSV antigen (Biodesign International) which is inactivated by gamma-irradiation. The antigen did not cause disease symptoms, and the mice continued to show good appetites and appeared healthy. On day 39, all 25 animals in the immunized group, including the three that had very low serum anti-RSV-F antibodies following oral immunization, showed significantly increased immune responses. Six of the animals showed 3–4 fold increases in antibody titers within the six-days post-boost. For all 25 mice, the mean endpoint serum IgG and IgA titers were significantly ($P \leq 0.05$) higher by 1.6 and 1.8-fold respectively (Figure 4B), compared to the orally-immunized (Figure 4A) mice, indicating a spike in antibody titer following booster immunization. None of the control mice developed measurable anti-RSV-F antibodies (Figure 4B). This suggests that mice were primed to recognize the F antigen as presented in RSV antigen following multiple rounds of oral immunization with the RSV-F subunit expressed in tomato fruit.

To determine the mucosal immune response to the transgenic fruit, intestinal extracts from both immunized and control mice on day 39 (6 days post-boost) were tested by ELISA for RSV-F-specific IgA titers. Among the 22 immunized mice with significant serum antibody responses, 18 also had significant RSV-F-specific intestinal IgA titers (Figure 4B). None of the control mice developed the mucosal IgA antibodies. These results indicate that oral immunization with transgenic tomato fruit elicited an F-antigen-specific mucosal IgA response.

Endpoint titers of the three sub-classes of RSV-F-specific IgGs were determined from control and from both orally-immunized (blood drawn on day 32) and boosted mice (blood drawn on day 39). The orally-immunized mice showed similar levels of RSV-

F-specific serum IgG_{2a}, IgG_{2b} and IgG_1 antibodies (Figure 4C). In contrast, the boosted mice showed significantly higher ($P \leq 0.05$) titers of serum IgG_{2a} and IgG_{2b} (endpoint titer 1:11000) compared to serum IgG_1 (endpoint titer 1:8000) (Figure 4D). Control mice did not develop anti-RSV-F IgG-subclass antibodies (Figure 4C, D). These results suggest that the transgenic-fruit-derived RSV-F antigen primed a mixed type 1–2 T-helper cell mmune response and further that this RSV-boost-induced response showed some bias towards the Th1-type (Mosmann & Coffman, 1989).

Discussion

RSV-F antigen expressed in transgenic tomato fruit has been shown here to produce serum and mucosal immune responses in BALB/c mice after feeding. This suggests the possibility of producing an edible vaccine against RSV and perhaps also other mammalian viruses utilizing the present approach. The preferentially fruit-expressed E8 promoter that is activated at the onset of ripening in tomato fruit (Lincoln et al., 1987) and the constitutively-expressed CaMV 35S promoter (Benfey et al., 1989) were used to drive RSV-F gene expression. The E8 promoter was as effective in ripening fruit as the CaMV 35S promoter (Figure 2), and transgenic plants containing the E8 promoter produced sufficient antigen to stimulate the production of serum and mucosal antibody responses in mice (Figure 4A, B). This result is consistent with recent studies showing that plant-derived microbial antigens, for example, the binding subunit of *Escherichia coli* heat-labile enterotoxin (LT-B) (Haq et al., 1995) and the cholera toxin B subunit (Arakawa et al., 1998), successfully serve as sources of edible vaccines in mice. The feeding of raw transgenic potato tubers expressing the LT-B subunit to human volunteers also resulted in their production of specific antibodies (Tacket et al., 1998), showing that a plant-based vaccine is feasible for human immunization.

The hypersensitivity to RSV infection associated with some inactivated and subunit vaccines is limited to individuals without prior exposure to RSV (Waris et al., 1997). After a single dose of a nonreplicating vaccine, antibody titers first rise in response to the antigen and then decline. When these individuals are challenged a second time, their immune systems produce a faster and more potent response. In RSV infections, this second exposure leads to a Th2-type response that is characterized by the infiltration of infected lung tissues with eosinophils (Srikiatkhachorn & Braciale, 1997a). The eosinophils produce compounds meant to slow the spread of invading extracellular pathogens, for example, bacteria, which in turn irritate lung tissues and lead to enhanced disease (Srikiatkhachorn & Braciale, 1997a). However, each subsequent exposure to the antigen induces the proliferation of more antigen-specific memory B cells and results in a more persistent and relatively high level of antibody production. Hence, individuals who have received multiple mucosal exposures to RSV antigens would be expected to have relatively high titers of RSV-specific antibodies and show a much less dramatic increase in antibody titer in response to RSV infection than individuals who received a single dose of inactivated vaccine. This is consistent with the results of the RSV boost of the orally-immunized mice presented here (Figure 4B).

An edible subunit-vaccine against RSV, produced by expressing the RSV-F protein in fruit, is a novel delivery system that has not been reported previously, therefore raising questions about its safety. However, it is well documented that an RSV-F subunit vaccine does not produce a hypersensitive response in children previously infected with RSV (Belshe et al., 1993; Tristram et al., 1993, 1994) and in those who have experienced two or more RSV seasons before vaccination (Paradiso et al., 1994). Further, another F-subunit vaccine has also shown protection against RSV in the elderly (Falsey & Walsh, 1996) and in children with cystic fibrosis (Piedra et al., 1996, 1998) and bronchopulmonary dysplasia (Groothuis et al., 1998). Thus, as a vaccine, the RSV-F subunit appears safe and well tolerated in humans.

Since the BALB/c mice used here produce a systemic immune response primed by the edible RSV-F vaccine, we will next analyze protection of test animals against RSV disease. Both the cotton rat and the BALB/c mouse are model systems used to evaluate immune responses and protective properties of candidate RSV-F vaccines (Walsh et al., 1987, 1997). Like humans, both animals are readily infected with RSV, develop similar lung pathology, recruit eosinophils to RSV-infected lung tissue after a single immunization with formalin-inactivated RSV, and show a Th1-type response to infection by wild-type RSV (Waris et al., 1997).

Reinfection with RSV is common throughout life. Therefore, development of serum immunity alone does not seem to be very protective, though prior RSV

infections can reduce the severity of the disease (Hall, 1992; Collins et al., 1996). However, multiple exposures of the mucosa to virus antigens during RSV infections have been hypothesized to 'mature' initial Th2-type responses to more protective Th1-type responses (Srikiatkhachorn & Braciale, 1997a). In this light, it is interesting to note that the edible RSV-F vaccine used here produced antibody titers with some bias toward a Th1-type response. Therefore, repeated stimulation of the mucosal system by a fruit-based edible vaccine eaten during the RSV season may provide protection against the disease.

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